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MINIREVIEW

Discovery and Development of New Antibiotics: the Problem of Antibiotic Resistance†

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Real clinical needs for new antimicrobial antibiotics derive from the emergence and dissemination of new opportunistic pathogens, especially in a growing immune system-debilitated host population. The significant health problems that occur as a result of infections with these rare or opportunistic pathogens is an outgrowth of the AIDS epidemic as well as the increasing prevalence of aggressive cancer chemotherapy and organ transplantation. Therapeutic needs can often be met by optimizing the use of existing chemotherapeutic agents. However, commonly prescribed antibiotics may not sufficiently cover these organisms, and the rapid spread or development of antibiotic resistance may compromise standard empiric treatment.

Indeed, the greatest threat to successful antibiotic coverage, and hence the driving force behind the search for new therapies, is the evolution and spread of antibiotic resistance. Resistance of common or resurgent pathogens to standard antibiotic therapies is a significant nosocomial problem and is of increasing importance in community-acquired infections as well. In the hospital setting, especially tertiary-care facilities (40), the incidence of drug-resistant gram-positive infections is increasing—notably among *Staphylococcus aureus*, coagulase-negative staphylococci, corynebacteria, and enterococci—while drug resistance in gram-negative organisms, including *Pseudomonas*, *Serratia*, and *Acinetobacter* species, continues to pose problems (20). The recent appearance of virulent strains of *Mycobacterium tuberculosis* that are resistant to multiple antibiotics in the AIDS, illegal drug addict, and prison populations has caused great alarm, posing a threat to the wider community and a potential resurgence of the disease (1).

Empiric therapy favors the use and hence the development of broad-spectrum agents and combinations (7), even though the underlying need may be for treatment of specific problem pathogens, such as *Pseudomonas* species or methicillin-resistant staphylococci. While future technological refinements may bring rapid diagnostic methods and lead to effective dosing with narrow-spectrum agents, the current strategy is to develop antibiotics with good pharmacological profiles and (relatively) broad spectra of activity, including activity against the problem pathogens. The effective spectrum of an antibiotic, for empirical dosing, is determined by the MIC for 90% of strains tested, which, when it is based on a sufficiently large sample size and is significantly different from the low end of the MIC range, is due to the existence of resistant organisms in the pathogen population. The antibiotic's spectrum is eventually defined by resistant organisms.

APPROACHES TO OVERCOMING RESISTANCE

How can the challenges of resistance emergence be met? Can novel antibiotics which have the necessary breadth of spectrum of activity for empirical use as well as a favorable pharmacological profile be designed or discovered and developed? How can this be rationally undertaken?

Design of novel analogs. One approach is to design or develop analogs of drugs that are already in clinical use and that have activity against resistant organisms. For example, modified erythromycins have been shown to overcome the resistance caused by the macrolide-lincosamide-streptogramin B (MLS) ribosome methylation mechanism (18). Certain lipophilic tetracycline analogs, such as minocycline, appear to be less susceptible to plasmid-borne tetracycline resistance mechanisms (34). Whereas the loss of outer membrane porins in gram-negative bacteria leads to decreased susceptibility to a variety of antibiotics (38), chemists have been able to modify drugs to take advantage of facilitated entry into the periplasm by means of solute-specific transmembrane channels and thus avoid cross-resistance with mutants that have lost their outer membrane porins. An example of this is the design of catechol-containing cephalosporins, which gain entry into gram-negative organisms, especially *Pseudomonas* species, via a catechol-scavenging mechanism (13, 39, 43).

Alternatively, many antibiotics derived from natural products are known, but they were not previously developed because of a lack of spectrum, possible toxicity, or other reasons. For example, 16-membered macrolides, such as spiramycin, are active against inducibly MLS-resistant strains (25), since they do not induce MLS resistance, although they are subject to it (50). Spiramycin is in use in France, and it, along with other 16-membered macrolides, may be of interest for development elsewhere. Pristinamycin, a naturally occurring mixture of streptogramins which is not subject to MLS resistance (2), has been in use in Europe, but its low solubility limits its usefulness. A semisynthetic mixture of water-soluble derivatives of the pristinamycin components (RP 59500) is being developed by Rhone-Poulenc (23). Other classes of antibiotic compounds might be candidates for development if an appropriate spectrum could be attained or if toxicities could be eliminated.

Drug combinations. When the molecular mechanism of resistance is characterized, it has been feasible to design specific therapeutic combinations on the basis of the antagonism of the major resistance determinants. This approach

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† This review is dedicated to the memory of John S. Wolfson.

has been reviewed by Labia et al. (29) and is exemplified by the introduction of amoxicillin-clavulanate combination therapy (5). In this case, amoxicillin, an antibiotic with a highly favorable profile (orally absorbed, broad spectrum, nontoxic, safe for pediatric use, and bactericidal), is paired with clavulanic acid, an inhibitor of the β-lactamases which are responsible for the majority of resistance to amoxicillin. Such combinations have meet with wide clinical success. While cephalosporinases that are poorly inhibited by clavulanate or sulbactam are rapidly arising (6), carbapenems such as SF-2103A (56), carpetimycins A and B (27), and the penem BRL-42715 (9), which can inhibit a variety of cephalosporinases (including clavulanate-resistant ones), have been reported to be useful in combination with broad-spectrum cephalosporins.

New therapeutic approaches. Without the development and introduction of new or improved antibiotics to ensure coverage of resistant organisms, empirical treatment must often rely on combination therapy. In the future, the use of adjunctive therapy with immune modulators such as granulocyte macrophage colony-stimulating factor and granulocyte colony-stimulating factor will certainly increase (21). Emphasis on vaccine development and use, for example, antihaemophilus and multivalent antipneumococcal vaccines, should be renewed. In the near term, wide acceptance of recommendations for the responsible use of antibiotics (33), with emphasis on the use of narrow-spectrum drugs over more broad-spectrum agents when appropriate, should lessen the selective pressure for resistance. Improved diagnostic methods that use DNA probe technology and the polymerase chain reaction will allow the rapid identification of infecting pathogens and may be able to pinpoint the existing resistance mechanisms in the pathogen, further enabling the practical use of narrow-spectrum or non-cross-resistant drugs. In any case, the direction of antimicrobial drug discovery must emphasize coverage of resistant organisms, ideally by pursuing mechanisms of action which do not favor the emergence of resistance.

Novel agents with new mechanisms. As discussed in a companion minireview (45), a target-directed approach has generally been taken to discover and develop new antibacterial drugs. One strategy for overcoming resistance has been to investigate inhibitors of novel targets, assuming that new chemical entities which are not susceptible to existing resistance mechanisms will be uncovered. Is this assumption reasonable? Will action on a novel target lead to susceptibility? Can targets that will lower the potential for development of resistance be chosen?

WHAT IS THE NATURE OF BACTERIAL RESISTANCE?

Two types of resistance. The nature of bacterial antibiotic resistance has been reviewed abundantly (for example, see references 27, 37, 39, 50), so we will not do so here. However, it is important to recognize the two overall types of antibiotic resistance which occur, i.e., "endogenous" resistance versus exogenous or "positive-function" resistance (3, 22). The endogenous type of resistance can arise in pure culture as a change or loss of function and is generally genetically recessive. Mutations that afford endogenous resistance to specific antibiotics are often deleterious to the organism in the absence of the antibiotic because they may involve changes in the target of the antibiotic, usually an essential enzyme or structure, or a change in permeability to the class of molecules including the antibiotic, thus reducing the amounts of useful or essential nutrients that enter the organism. Such resistant mutants are unlikely to compete well for survival in the absence of drug. Thus, they may not contribute to the overall susceptibilities of pathogen populations unless antibiotic selection continues (because of overuse, for example) and the organisms acquire compensatory mechanisms (mutations) which increase their fitness. While endogenous resistance may not be evident in pretreatment isolates, it can arise and be selected for in the patient, and this may compromise therapy with certain antibiotics, for example, rifampin. Endogenous resistance can be modeled in the laboratory by direct selection of mutants resistant to drug at concentrations greater than the MICs and through enrichment by rounds of challenge with the drug at concentrations that are at or slightly greater than the MIC.

The second type of resistance, positive-function resistance, generally arises through horizontal transmission of exogenous, evolved, genetically dominant functions. This positive-function resistance may be less unstable than endogenous resistance in the absence of selection, in that it involves the addition of functions, such as the creation of antibiotic-detoxifying enzymes rather than a change in existing enzymes or structures. It is this type of resistance which appears to be the major contributor to the baseline resistance (MICs for 50 or 90% of isolates tested) of the pathogenic species and which will be recognized in the antibiotic susceptibility pattern of the pretreatment isolate. This type of resistance cannot generally be modeled in a pure culture of a naive susceptible pathogen, except when it occurs by mutational turn-on of cryptic genes, such as cryptic lactamases in gram-negative organisms, and multiple antibiotic resistance (Mar) functions (8).

Bryan (3) contrasted positive-function resistance with "bacterial persistence" and included, as well as mutation to true resistance (the ability to grow in the presence of a drug), those changes or states that allow organisms to survive but not necessarily grow in the presence of a cidal drug. Such persistence or tolerance may affect the MBCs but not the MICs for posttreatment isolates. The stabilities of the changes that lead to persistence in the absence of selection are not clear. Bacterial persistence can lead to therapeutic failure for a number of drugs, notably, penicillin (19, 47), and is likely to play a role in situations in which complete eradication of infection is required, for example, in immune system-compromised or severely debilitated patients. With certain antibiotics, the development of endogenous resistance is so likely that it precludes their use as single agents.

What is it about antibiotics which make them prone to endogenous resistance development? Table 1 lists antibacterial chemotherapeutic agents (both natural products and synthetic compounds) and indicates the clinically significant forms of endogenous and exogenous resistance to which they are subject. The antibiotics which are in broad clinical use (penicillin, cephalosporin, tetracycline, macrolide-lincosamide, chloramphenicol, aminoglycoside, and glycopeptide classes) are generally not subject to the occurrence of high-level endogenous resistance because of a loss or change of normal function that is consistent with virulence or stability in the absence of selection. With the continued use of these antibiotics, however, positive-function resistance determinants enter pathogen populations, and their prevalence is the major threat to the continued success of these antibiotics.

Other natural products, such as rifamycins, novobiocin, or fusidic acid, are subject to significant development of high-level endogenous resistance, generally via mutation of the antibiotic target, thereby precluding their use as single

TABLE 1. Clinically relevant forms of endogenous and exogenous resistance to antibacterial agents[a]

Example, class	Target class: molecular target	Clinically significant:	
		Endogenous resistance	Positive-function resistance
Penicillin, cephalosporin β-Lactam	Cell wall synthesis: multiple PBPs (peptidoglycan synthetic enzymes)	Multiple changes in PBPs (*Streptococcus pneumoniae*)	β-Lactamases; new penicillin-resistant PBPs (staphylococci)
Vancomycin Glycopeptide	Cell wall synthesis: terminal D-Ala–D-Ala of pentapeptide		Membrane protein; ligase with altered specificity
Fosfomycin Phosphonic acid	Cell wall synthesis: PEP enoyl transferase	Loss of permease, but nonvirulent	Inactivating enzyme
Tetracycline Tetracycline	Protein synthesis: 30S ribosome subunit		Active efflux; ribosome "protection"
Chloramphenicol Chloramphenicol	Protein synthesis: 50S ribosome subunit		Antibiotic-inactivating enzymes; efflux systems
Erythromycin Macrolide	Protein synthesis: 50S ribosome subunit		Methylation of 23S rRNA; inactivating enzymes; active efflux?
Kanamycin Aminocyclitol	Protein synthesis: 30S ribosome subunit	Mutation linked to *nek* or *rpsQ* clinical relevance unknown	Antibiotic-modifying and -inactivating enzymes
Fusidic acid Steroid	Protein synthesis: elongation factor G	Single mutation prevents drug binding to elongation factor G	Chloramphenicol acetyltransferase sequesters fusidic acid
Rifampicin Rifamycin	RNA synthesis: RNA polymerase β′ subunit (*rpoB*)	Single *rpoB* mutation prevents drug binding	None described
Novobiocin Coumarin	DNA synthesis: DNA gyrase B subunit	Single mutation can lower affinity relative to substrate ATP	
Norfloxacin Quinolone	DNA synthesis: DNA gyrase-DNA complex	Single mutations in *gyrA* or *gyrB*; permeability changes	None described
Trimethoprim Dihydrofolate analog	Folate synthesis: dihydrofolate reductase	Single mutation lowers affinity relative to substrate; also derepression	Metabolic bypass; resistant dihydrofolate reductase

[a] PBPs, penicillin-binding proteins; PEP, phosphoenolpyruvate.

agents. They have thus been developed for narrow applications, generally in combination therapy.

What distinguishes the two drug types? We hypothesize that, aside from the development of endogenous resistance due to permeability changes, drugs that are susceptible to clinically significant endogenous resistance development, for example, rifampin and novobiocin, are those which interact with a single gene product, in which single mutations are more likely to prevent drug binding and thus can lead to high-level resistance at a high frequency. Drugs having a low likelihood for the development of endogenous resistance, such as the β-lactams, are those that interact with multiple intracellular molecular sites, whose structures are determined by multiple genes. Single mutations consistent with survival may not reduce the required interaction of drug and target sufficiently for significant resistance. In the case of many inhibitors of protein synthesis, for example, the actual molecular target appears to be (or at least to contain) rRNA (12). Since most bacteria have multiple copies of rRNA genes, single mutations would be insufficient to prevent antibiotic binding.

FLUOROQUINOLONES: WHERE DO THEY FALL?

The fluoroquinolones, such as norfloxacin, ciprofloxacin, and ofloxacin, synthetic drugs that have been introduced relatively recently, are not (yet) subject to horizontally transmitted positive-function resistance (10). These agents are targeted at a DNA gyrase-DNA complex (42). In the laboratory, single missense mutations in the gyrase A (or gyrase B) subunit of *Escherichia coli* can be selected. These single missense mutations afford increased resistance to fluoroquinolones by a yet to be defined molecular mechanism, generally increasing the MICs 2- to 10-fold (11, 52). Mutations that reduce fluoroquinolone permeation can also be obtained both in the laboratory and in the clinic (4, 26). For organisms that are initially highly susceptible, such as *E. coli*, single mutations give MICs that are within the "susceptible" range (by in vitro MICs). In intrinsically less susceptible organisms, such as staphylococci and *Pseudomonas* species, the MICs following a single mutation may exceed the therapeutic breakpoint. That is, the initial MIC for *E. coli* is low enough so that single mutations do not generally increase the MICs above therapeutically achiev-

able levels, whereas for staphylococci, initial MICs for susceptible organisms are high enough that a similar fold increase in the MIC may compromise therapy. Current efforts to obtain fluoroquinolones with increased potencies against gram-positive organisms may address this problem. A sobering note is that even when the initial increase in the MIC for highly susceptible organisms is below the breakpoint, continued selective pressure can lead to the emergence of higher-level resistance via additional mutations, as has been seen in clinical *E. coli* isolates in Spain, France, and Japan.

Clinically, fluoroquinolone resistance appears to involve mutations both in DNA gyrase and in the pathway for permeability (52). While reports of therapeutic failures with fluoroquinolones exist, these are relatively rare and do not appear to have affected baseline MICs except in specific hospital settings. However, it is clear that important nosocomial fluoroquinolone resistance is occurring (53) and may be occurring more rapidly than was the case with earlier broadly used antibacterial agents which were subject to the spread of positive-function, high-level resistance. It is interesting that multistep high-level endogenous fluoroquinolone resistance can occur during therapy and may be approximated in vitro by sequential passage through increasing levels of drug (32). It is not known whether this is a quinolone-specific phenomenon, perhaps reflecting the mutagenicity of these compounds (17), or whether this places fluoroquinolones in a continuum of frequency at which successful mutations that increase resistance can occur.

CAN TARGETS FOR WHICH ENDOGENOUS RESISTANCE WILL NOT ARISE BE RATIONALLY CHOSEN?

A conclusion from the above discussion is that antimicrobial agents whose receptors are determined or encoded by multiple genes may have a better chance of escaping significant endogenous resistance than agents with single gene targets. Successful ones are natural products which were (most likely) evolved for that purpose (44). In general, these antibiotics were discovered through broad screening of natural products for nontoxic, broad-spectrum, potent antibacterial agents. Continued screening of natural products for inhibitors of essential microbial macromolecular synthetic pathways is therefore still indicated for the discovery of new, evolved, multitarget antibiotics.

Is there a rational way to develop multitarget therapies? One approach is to design a dual-action molecule that has two different targets, such as the Roche quinolone cephalosporin Ro23-9424 (16). In theory, cephalosporin-susceptible cells would be inactivated by the cephalosporin moiety; if a cephalosporinase were present, it would liberate the quinolone moiety, which could penetrate the cytoplasm and reach its target. While the details of this lactamase activation have not been proven, it is clear that the compound both binds to penicillin-binding proteins and inhibits replicative DNA synthesis (16). Perhaps a quinolone derivative could be designed to contain a moiety which inhibits a second cytoplasmic target in addition to DNA gyrase. For that matter, it is possible that two different enzymes specific to bacteria might be inhibited by the same ligand. Are there sets of essential targets, in addition to the penicillin-binding proteins, which could be inhibited by the same molecule?

One successfully exploited avenue has been the development of combinations of inhibitors, such as sulfamethoxazole-trimethoprim, which inhibit different steps in the folate synthetic pathway. Such a double-blockade combination, fludalanine-pentizidone (51), which inhibits the cytoplasmic D-Ala–racemase and D-Ala–D-Ala synthetase steps in cell wall synthesis, was investigated at Merck, but development was discontinued because of non-mechanism-based toxicity.

These combination double-blockade agents exemplify the situation in which inhibition of a single enzyme in a pathway subject to mass action may lead to a buildup of substrate and adaptation to the inhibition. Unless the enzyme is completely blocked, internal pools could alter to accommodate inhibition. If the pool levels are otherwise limited, then mutations which up-regulate enzymes in the pathway might lead to resistance. It is for this reason that a double blockade of the pathway (cell wall pathway by fludalanine-pentizidone; dihydrofolate pathway by trimethoprim-sulfamethoxazole) is necessary (15).

The advent of sophisticated protein structural and analytical methodologies should also be exploited for rational drug development. It has a definite place in the development of drugs for targets not subject to resistance development and for which substrate-competitive inhibitors are desirable, for example, mammalian enzymes. What role can it have in the development of novel antimicrobial agents? Can detailed study of a purified target protein be directed to model inhibitors whose interaction with the target is unlikely to be prevented by a single mutation?

To avoid the likelihood of developing endogenous resistance, it would be advantageous if alteration of the target enzyme to drug insusceptibility were neither a frequent event nor compatible with survival (or virulence). That is, unlike the target modifications in human immunodeficiency virus reverse transcriptase that cause resistance to zidovudine (31) and that are compatible with normal functioning, the desired drug target should be difficult to alter to drug insusceptibility without compromising normal activity.

An empirical approach would be to obtain inhibitors of a chosen target and then to select for mutations in the target protein which render it insusceptible to the inhibitor. A second round of inhibitor screening could be done, requiring activity versus both the original (susceptible) and mutant (resistant) targets. This could be extended through several parallel or sequential cycles, with the ultimate inhibitor being unlikely to be subject to target-based resistance. The rational design of inhibitors for which endogenous resistance is unlikely to arise is conceivable, although it is an extremely long-term goal. To approach this design, a suitable target protein, amenable to protein crystallographic analysis, would be chosen and inhibitors would be obtained by specific screening or structure-based design (28). The structure of protein-inhibitor complexes could then be determined, with particular attention given to inhibitor-enzyme contacts. Directed substitution of amino acids in the inhibitor-binding region of the protein would give information about the changes from the wild type that are compatible with growth and the degree to which susceptibility or resistance to known inhibitors is altered. Targets resistant to inhibition could be selected through standard genetic procedures as well, giving insight into the most probable changes. With structural analysis of these altered proteins, it should eventually be possible to design compounds which are capable of inhibiting many if not all of the possible active structures of the target.

Would an inhibitor that is competitive with a small molecule substrate (an active-site inhibitor) be less subject to alteration to drug insusceptibility than one that interacts with an allosteric site on an enzyme? One would expect that

constraints on changes at the active site are greater than those at other sites in an enzyme, since an active site must retain affinity with the correct small molecule (substrate) while reducing affinity for the inhibiting small molecule. Mutations that lead to the use of alternate substrates would increase the number of viable possibilities.

Such a model is difficult to substantiate, since most evidence is anecdotal. Rifampin and fusidic acid are examples of inhibitors that are not competitive with small molecule substrates, and in both cases, single mutations to high-level resistance can occur. They are relatively stable (in pure culture) and can compromise therapy. For coumermycin, a competitive inhibitor of the ATPase of the β-subunit of DNA gyrase, 5 to 30% of spontaneous resistant mutants are temperature sensitive for growth (41), indicating that resistance can arise in different ways on the same target and that while some of these changes are inconsistent with growth, the majority have no obvious deleterious effects. An interesting case of the success of an active-site inhibitor of a single enzyme target is that of the natural product fosfomycin. Fosfomycin-resistant mutants with an altered target enzyme, phosphoenolpyruvate enoyl transferase, have been isolated after mutagenesis (48). These mutants are temperature sensitive, indicating that the change to resistance may be incompatible with normal enzyme function. In the laboratory, fosfomycin resistance arises rapidly from the loss of a permease, but these permease-resistant mutants are generally of low virulence (54). In the clinical setting, while fosfomycin permease-resistant organisms have been implicated in urinary tract infections (24), this endogenous resistance does not appear to compromise its general effectiveness in the treatment of acute-phase urinary tract infections (37). Plasmid-borne positive-function resistance to fosfomycin has been seen in enterobacteria, especially *Serratia* species (35), and in staphylococci (14).

For the rational design of inhibitors, active-site competitors are more amenable to modeling and prediction. It is instructive to note that with dihydrofolate analog inhibitors of dihydrofolate reductase such as trimethoprim (46) or the hydroxyphenylazopyrimidines (55) which are inhibitors of certain bacterial DNA polymerases competitive with dGTP, mutations to resistance may occur at sites adjacent to active sites, preventing the binding of inhibitor while not interfering with substrate recognition. Generally, these inhibitors are more bulky than the natural substrates, preventing access to the active site but extending beyond it, with occupancy by the normal substrate preventing inhibitor binding. It appears that for such inhibitors for which the inhibitor binding site extends beyond (includes more contact than) the substrate interactive site, single base changes can lead to resistance. Thus, design of less bulky inhibitors might reduce the opportunity for successful alteration to resistance.

In this regard, recent observations on inhibitors of human immunodeficiency virus type 1 reverse transcriptase may be relevant. Human immunodeficiency virus isolates that are resistant to the nucleoside analog zidovudine can be isolated from patients after 6 months of zidovudine therapy (30). These isolates have multiple mutations in the reverse transcriptase gene (31), although the complete molecular mechanism of resistance is not yet clear. While data for similar studies that follow the susceptibilities of isolates after initial therapy with dideoxyinosine have not been published, early reports indicate that resistance development during dideoxyinosine therapy may be less evident than that during zidovudine therapy (36, 49). Because dideoxyinosine is less bulky than either zidovudine or the natural nucleoside precursors of reverse transcriptase substrates, it is possible that the apparent difference in resistance selection reflects a relative difficulty in altering the enzyme to dideoxyinosine insusceptibility without the loss of fitness. Because these results are preliminary and other explanations are possible, further studies will be needed to determine the molecular mechanisms and relative frequencies of resistance development to zidovudine and dideoxyinosine during therapy.

CONCLUSIONS

Enhancement of current drug classes to overcome existing positive-function resistance is an active area of research. New drugs not subject to existing resistance mechanisms may be designed by focusing on the discovery and development of novel chemical entities to inhibit single or even multiple enzyme targets, but we must be prepared to attack the problem of endogenous resistance development early in the discovery and design phases. In antiviral therapy with drugs targeted at specific enzymes, it has become the practice to give sequential or simultaneous dosing of different drugs, just as it has become necessary to do so in the treatment of mycobacterial infections when the multitargeted broad-spectrum drugs are not successful. Combination therapy is clearly an accepted part of clinical practice, and rationally designed combinations such as sulfamethoxazole-trimethoprim and amoxicillin-clavulanate are well accepted. Codevelopment of novel single-enzyme inhibitors as combinations designed to overcome endogenous resistance development to each single entity should also be pursued.

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